IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Pablo STEINBERG, et al.

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For: Method for conducting non-invasive early detection of colon cancer and/or of colon cancer precursor cells

Art Group: 1637

Examiner: Mark STABLES

DECLARATION UNDER 37 C.F.R. §1.132

I, Pablo Steinberg, a citizen of GERMANY hereby declare and state:

1. I have a degree in biochemistry that was conferred upon me by the University of Buenos Aires, Buenos Aires, Argentina, in 1985. The degree is the PhD.

2. I have been employed by the University of Mainz (Mainz, Germany) between October 1985 and September 1998, by the University of Potsdam (Potsdam, Germany) between October 1998 and March 2008 and by the University of Veterinary Medicine Hannover (Hannover, Germany) since April 2008, and I have had a total of 12 years of work and research experience in researching and developing of methods for conducting non-invasive early detection of colon cancer and/or of colon cancer precursor cells.

3. I am one of the named inventors in the above-captioned patent application. I am familiar with the patent application.

4. If a patent issues from this application and if it is decided by the assignee to pursue a commercial product falling under its claims and if such a commercial product is approved by FDA and sold in the US, then under German law, I and the other inventors may receive some compensation derived from such sales. I am not being directly compensated for my work in connection with this Declaration.

5. I and/or those under my direct supervision and control have conducted the method of the present application.

6. At the time of the present invention a multitude of signaling pathways using different marker genes are known which are all associated with cancer. It was known that oncogenes and tumor suppressors depend on one another for their selective advantage and that they affect multiple pathways that intersect and overlap. On the basis of gene mutations the signaling pathways are reprogrammed during the genesis of cancer and the cells react with uncontrolled overgrowth.

7 Known signaling pathways are the Wnt pathway and the MAPK pathway as well as the further important signaling pathways for example phosphatidylinositol 3-kinases/Akt family way, the TGFß way, the $NF_{\kappa}B$ way and the tumor protein p53 way. The named and further pathways are cross-linked in cells and have an effect on each other. The proteins being receptors, enzymes, adaptors, inhibitors, transcription factors and so on operate together in each signaling pathway and are coded by a multitude of genes.

8. For an analysis of the Wnt pathway a lot of genes could be relevant, namely genes which code for components of this signaling pathway (for example Wnt, Frizzled, LRP5/6 Dishevelled, Axin, GSK-3ß, Apc, ß-Caterin and Tcf/Lef) as well as genes coding for gene products which interact with the components of the Wnt pathway (for example α-Catenin, p120 and E-Cadherin) and furthermore target genes (for example cyclin D1 or c-myc), which stimulate the transcription by the ß-Catenin/Tcf/Lef complex.

9 For an analysis of the MAPK signaling pathway genes are relevant which code for the following components: receptor tyrosine kinases (for example EGFR and PDGFR) growth factors, Ras, Grb2, SOS, Shc, GTPase activating proteins (GAPs), p85, p110, A-Raf, C-Raf, MEK1, MEK2, Erk1, Erk2, Mnk1, p90, RSK, Ets, Elk-1 and SAP-1, target genes (for example c-fos, c-jun, cyclin D1 and c-myc) and genes coding gene products which interact with Ras or with the receptor tyrosine kinases (for example PIP3, Akt, NF-1, JNK, SAPK, p38 MAP kinases and more) For the other signaling transduction pathways there could be named numerous relevant genes as well. A lot of components of the signaling pathways are unknown until now.

10. At the time of the present invention experts combined genes in different marker panels for mutation analysis of tumor tissue or stool samples, which were found in patients

having colon carcinomas. These marker panels were of modest success.

11. For example Salahshor et al. (Genes Chromosomes and Cancer, Volume 26, Number 3, November 1999, pages 247-252; document cited by the Examiner) show the frequency of mutations of the genes APC, K-Ras, TP53 and TGFßR2 in MSI-H tumors. The mutation frequency was 86.4% for TGFßR2 and between 4% and 23% for the others. In view of MSS tumors no TGFßR2 mutations were found but a 35%, 57% and 60% frequency of mutations in cases of K-Ras, APC and TP53. A total detection rate of the analyzed carcinomas was not published.

12. Davies et al. defined the gene marker B-Raf, which mutated in 86% of melanomas (Nature, Vol. 418, Number 20, August 2002, pages 949-954; also a cited document by the Examiner). However, this gene marker showed in colon carcinomas only a 15% mutation frequency.

13. The idea and subject matter of the present invention was now to find an improved method for an analysis of colorectal cancer (CRC) at a very early stage. The present invention shows a method using a marker panel having defined primer pairs for the four genes APC-and ß-Catenin (also known as CTNNB1) as well as K-Ras and B-Raf, which cover the Wnt and Ras-Raf-MEK-MAPK signaling pathways.

14. Surprisingly, we found that the combination of the analysis of these four defined marker genes of colon cancer are able to allow an efficient detection of CRC in a very early stage (UICC I), whereby the analysis of two genes of the one signaling pathway - the Wnt pathway - are essentially combined with the analysis of two genes known to react of an other signaling pathway - the MAPK pathway.

15. It was not obvious from the cited prior art that the claimed selection of these four genes - APC, ß-Catenin, K-Ras and B-Raf - is able to detect a high proportion of the very early stage of colon cancer (UICC stage I).

16. This selection bases on the own cognition of the inventors that components are detected from at least two signaling transduction ways for the non-invasive early detection of colon cancer or intestinal cancer precursor cells because the colorectal carcinoma can be developed by different mechanisms, which include the permanent activation of signaling ways, the suppression of tumor suppressor genes or the cut (break down) of intracellular

repair mechanisms.

17. It was a further cognition of the inventors that two genes per signaling way have to be analyzed, which are able by a mutation to give a permanent activation of the each signaling pathway. This key function has to be realized from both genes of the respective pathway. In the case that APC on the Wnt way mutated, the gene product is a C-terminal truncated protein, which is not able to form a complex with Axin, GSK-3ß and ß-Catenin. That means, the ß-Catenin is not removed but is concentrated in the cells and translocated within the nucleus. It releases there the transcription of target genes together with the transcription factors Tcf/Lef. This increases cell proliferation.

18. On the other side, in the case that the ß-Catenin mutated the removing of the molecule is blocked with the same result for the cell. This explanation is also valid in the case of the MAPK signaling pathway, a mutation in K-Ras gene or B-raf gene leads to a permanent activation of the signaling pathway.

19. Second, it was furthermore not obvious that the amplified gene regions in which the mutation analysis is made and which are covered by the presented primer sequences are sufficient to detect a high proportion of the early stadium of cancer (UICC stage I).

20. The APC gene has a length of about 139000 bp, the B-Raf gene has a length of 190000 bp, the ß-Catenin gene about 41000 bp and the K-Ras gene more than 45000bp. In view of the multitude of possibilities for a gene region selection it was not obvious that the defined combination of the selected sequences will allow a sensitive detection of an early colorectal cancer stadium.

21. In this connection I refer to my enclosed publication in Cancer Epidemiology 33 (2009) 123-129. The aim of the study was to use a 4-gene marker panel of the application covering the Wnt and Ras-Raf MEK-MAPK signaling pathways to determine the percentage of sporadic colorectal carcinomas (CRC) alone and/or in combination with microsatellite instability (MSI) and to compare the sensitivity of the used gene marker panel of the application with that known gene marker panel (see page 124, left column, 3rd paragraph, [24]).

22. The percentage of CRC carrying at least one of the four above mentioned in a mutated form alone and in combination with MSI was determined in 50 sporadic CRC

samples by using the defined primer panel.

23. The used primer panel in the cited publication was in accordance with the present application SEQ ID No. 15 and 10 for APC in combination with SEQ ID No. 13 and 14 for ß-Catenin covering the Wnt signaling pathway as well as SEQ ID No. 11 and 12 for K-Ras and SEQ ID No. 17 and 18 for B-Raf covering the Ras-Raf-MEK-MAPK signaling pathway.

24. The known U.S. National Cancer Institute microsatellite panel used for comparison is described on page 124/125, item 2.5. The corresponding primer sequences are listed in Table 1.

25. Table 2 describes patients and tumor characteristics.

26. In Table 4 on page 126 it is shown that about 80% of early stage colorectal carcinomas (CRC) - UICC-stage I - are detectable using the new primer panel of the present invention. There the sensitivity of CRC detection (%) can be seen. As it can be taken from it in difference to stage II carcinomas and stage IV carcinomas the combination of the claimed marker panel with the well known MSI analysis did not enhance the percentage of tumors being positive in the case of stage I tumors. It is pointed out that the claimed method using the defined primer of the four marker genes analyzed represent early and initiating mutations in CRC development with high accurateness.

27. This was very surprising. Therefore the claimed method is an efficient method for detection of early and initiating mutations in CRC development.

28. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing there from.

Date: 14 06 2010

Pablo Steinberg

Encl.

Cancer Epidemiology 33 (2009) 123-129